AD_____

Award Number: W81XWH-FF-F-FHH

TITLE: Ô@æ&æ&c^¦ã ã *ÂUPÚGÂæ Âæ Áà[ç^¦ÁV@¦æ]^˘ à&Áæ¦*^ơ§ ÁÒ¦^æ ơÔæ} &^¦

PRINCIPAL INVESTIGATOR: Zæ&@à^ÁPæ¦ơ¦æ}

CONTRACTING ORGANIZATION: Y^•ơXã*ã ãæÁW¦ã&^¦•ã ÁÜ^•^æ&@ÁÔ[¦][¦æã}
T[¦*æ}ơ¸}ÊÁXÁ GÎ Í€ÎÁ

REPORT DATE: Ø^à¦ æ¦ÁGFG

TYPE OF REPORT: Annual Ù˘{ { æ¦

PREPARED FOR: U.S. Army Medical Research and Materiel Command
                Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release; distribution unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE (DD-MM-YYYY) 01-02-2012	2. REPORT TYPE Annual Summary	3. DATES COVERED (From - To) 15 JAN 2011 - 14 JAN 2012
4. TITLE AND SUBTITLE Characterizing SHP2 as a Novel Therapeutic Target in Breast Cancer		**5a. CONTRACT NUMBER**
		5b. GRANT NUMBER W81XWH-11-1-0133
		5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S) Zachary Hartman E-Mail: zhartma1@mix.wvu.edu		**5d. PROJECT NUMBER**
		5e. TASK NUMBER
		5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) West Virginia University Research Corporation Morgantown, WV 26506		**8. PERFORMING ORGANIZATION REPORT NUMBER**
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012		**10. SPONSOR/MONITOR'S ACRONYM(S)**
		11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Over the course of the past year, our purpose has been to identify important binding components that contribute to Shp2 substrate selectivity in an effort to contribute knowledge toward designing a selective Shp2 inhibitor. We have confirmed that the peptide based on a Shp2 substrate inhibits Shp2 and not the close homologue Shp1. Molecular modeling shows that point mutations at acidic residue do not always confer defects in binding. Results suggest that the -1 and -4 position acidic residues are critical for binding. A surprising finding from the modeling showed that the -2 acidic amino acid inhibited interactions of the peptide with the active site of Shp2. The knowledge that we gleaned from these studies was used to predict and characterize a substrate for Shp2, focal adhesion kinase. We intend to utilize this knowledge as we move forward in studying Shp2 action on HER2 through mutagenesis.

15. SUBJECT TERMS
phosphatase, inhibitor, kinases, receptors, modeling

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	**b. ABSTRACT** U	**c. THIS PAGE** U	UU	15	**19b. TELEPHONE NUMBER** (include area code)

Table of Contents

Introduction

This project relates to the non-receptor protein tyrosine phosphatase Shp2. It has been suggested to be an important mediator of signaling in a variety of forms of cancer (Mohi & Neel, 2007) and especially cancer of the breast (Zhou, et al, 2008). The overall purpose of this project is to characterize Shp2 as a therapeutic target in breast cancer, with specific goals related to understanding the nature by which Shp2 is able to identify its substrates in vivo and then characterizing an inhibitory peptide based on this understanding. It is intended that the knowledge gained from this line of study will promote the field's ability to create pharmacological agents against Shp2 in the future, which has been notoriously difficult to accomplish selectively (Chen et al., 2006; Hellmuth et al., 2008; Zhang et al., 2010). The research conducted thus far on Shp2 selectivity has yielded mixed results, with one study showing a consensus for selective substrates of Shp2 (L. Ren et al., 2011), but unfortunately this work gave little insight into the specific molecular interactions that must be in place for Shp2 to recognize a specific substrate; therefore, we feel this line of work has the potential to yield significant value to the rational design of anti-Shp2 agents.

Body

Substrate-based inhibitory peptide is selective against Shp2 compared to Shp1.

Over the course of a year, we have pursued the characterization of Shp2 selectivity in a variety of ways. First, we wanted to address one point that was unclear at the time of submission of our preliminary data, whether the peptide that was developed based on endogenous Shp2 substrates (sequence DADEpYL) was specific for action against Shp2 compared to a close homologue, Shp1. In published studies, Shp1 tends to show up as one of the major off-target hits for pharmacological agents (L. Chen et al., 2006; Hellmuth et al., 2008), so this was an important question to answer. We utilized the fluorescence-based difluoromethylumbelliferryl phosphate (DiFMUP) substrate, which pilot studies suggest is a better compound to assay compared to the use of paranitrophenol phosphate. This showed clearly that our peptide was unable to inhibit Shp1 to the extent of Shp2 (Figure 1), and indeed over the course of the assay the inhibition appeared unchanged, which was surprising given that we expected that in short order Shp2 would dephosphorylate and inactivate the inhibitor.

Molecular dynamics simulations corroborate and refine the initial docking data

The substrate peptide has been previously created and docked *in silico*, as presented in the preliminary data of the initial application. Since that time we have worked to refine this data and predict the most important peptide to active site interactions that constitute specific binding. To this end we took the docked peptide and mutated it *in situ* to make 5 peptides in total, and they included the following sequences: DADEpYL, DADEYL, DAAEpYL, DADApYL, and DAAApYL (pY represents phosphotyrosine, which was treated as fully deprotonated for the simulations). Each of these systems was run through molecular dynamics for 500 picoseconds before visual and computational assessment of binding. Energy decomposition was run on the systems in order to compare the overall favorability of the peptide binding (Table 1). Overall, what we have found so far has been surprising, with the data suggesting that the presence of a -2 acidic residue in the substrate peptide actually destabilizes the binding compared to mutation to alanine at that site. This was found to be true comparing the binding of the DADApYL peptide to DAAApYL (Figure 3 vs. Figure 4) as well as in comparing the DADEpYL to DAAEpYL peptides (Figure 2 vs. Figure 5). Interestingly, this implies that the DAAEpYL is the most optimal binding partner for Shp2 of the peptides so far studied, suggesting that it may make an even better inhibitor *in vitro*. Synthesis of the DAAEpYL peptide is now underway to evaluate this implication biochemically.

Molecular dynamics simulations have certainly helped to refine the initial docking poses, with the initial hypothesis being that the pY -1 and -4 position acidic amino acids will bind a pair of lysines adjacent to the active site (K364 and K366), and the pY -2 acidic amino acid will bind a small group of polar amino acids (Y279 and N281) on the opposite end of the active site. Simulations suggest that the -2 position seems to move away from any prominent side chain interactions, favoring interaction with water. This may provide an explanation for the predicted loss of binding of the peptide compared to when the -2 position is mutated to alanine, as competing binding to water can provide a significant driving force away from the active site. The -1 and -4 amino acids behaved generally as predicted, consistently forming strong ionic interactions with lysines 364 and 366, along with a previously unseen interaction of the acid groups to arginine 362. This is an important refinement since this arginine residue has been predicted to play a significant role in improved selectivity of inhibitors (Hellmuth et al., 2008). Tyrosine 279, which was not found to bind the peptide in the docked structure, revealed an

ion-dipole interaction with the -4 position acidic residue of the substrate peptide after simulation. When the -1 acidic residue was removed, many of these ionic interactions disappeared, with a consequent deficiency in the predicted stability of binding (Table 1). When the -2 position aspartate was mutated, we saw a significant stabilization of the peptide binding, typically providing approximately an extra 10-20 kcal/mol stabilization of the binding interaction. We feel this is due to the hydrophobic sidechain of alanine seeking to remove itself from the solvent, which forces the other acidic residues closer to their positively-charged binding partners. In total, this task has proven fruitful so far, and we intend to move forward in both the biochemical demonstration of binding and in predicting the impact of other *in silico* mutations, such as removal of all acidic residues, removal of the -4 position aspartate, and rearrangement of the acidic residues.

Prediction of a different Shp2 substrate from binding data

Shp2 is known to exert significant control over cell motility (Inagaki et al., 2000; Manes et al., 1999), a process that is important for metastasis in breast cancer. In order to test our model for binding selectivity based on the known substrate sequence of HER2, we predicted that Shp2 would bind to and act upon focal adhesion kinase (FAK). FAK is a critical mediator of a variety of cell processes, especially adhesion and migration. More importantly within the scope of this project, the most important phosphotyrosine site on FAK, Y397, is extremely similar in terms of its surrounding amino acids to the HER2 and EGFR substrates. Thus, we expected this site to be important for binding of Shp2. Full-length substrate-trapping Shp2 was able to associate with FAK after adhesion of fibroblasts to fibronectin, and we found this to be both direct (through far western analysis(Figure 6c)) and dependent upon phospho-Y397 (through mutagenesis and an *in vitro* phosphatase assay (Figures 6a, 6b, and 6d)). This line of study gives evidence that binding selectivity of Shp2 is indeed based upon the interactions of a phosphotyrosine's surrounding acidic residues. In addition, exploring the cellular mechanism by which Shp2 controls migration will allow us to understand in more detail why Shp2 is an effective drug target in the treatment of breast cancer. We feel that the experience gained through this line of experimentation will also be beneficial in moving forward with the HER2 mutagenesis, in addition to aiding in proof of concept that our Shp2 selectivity model can be predictive.

In the course of our study of FAK we were able to develop a construct introduced into FAK -/- fibroblasts that possessed substitutions of acidic residues for asparagine at the the -1 and -2 positions (2 aspartate to asparagine double mutant FAK), which proved convenient compared to HER2 since the necessary parameters for subcloning mutant FAK into a retroviral construct had already been worked out, and in theory it provided an extremely similar system to HER2 to perform essentially the same line of study as laid out in task 1 of the statement of work. Characterization of Shp2 binding to the mutant FAK is underway, and we have also obtained all the necessary tools to perform mutagenesis on HER2.
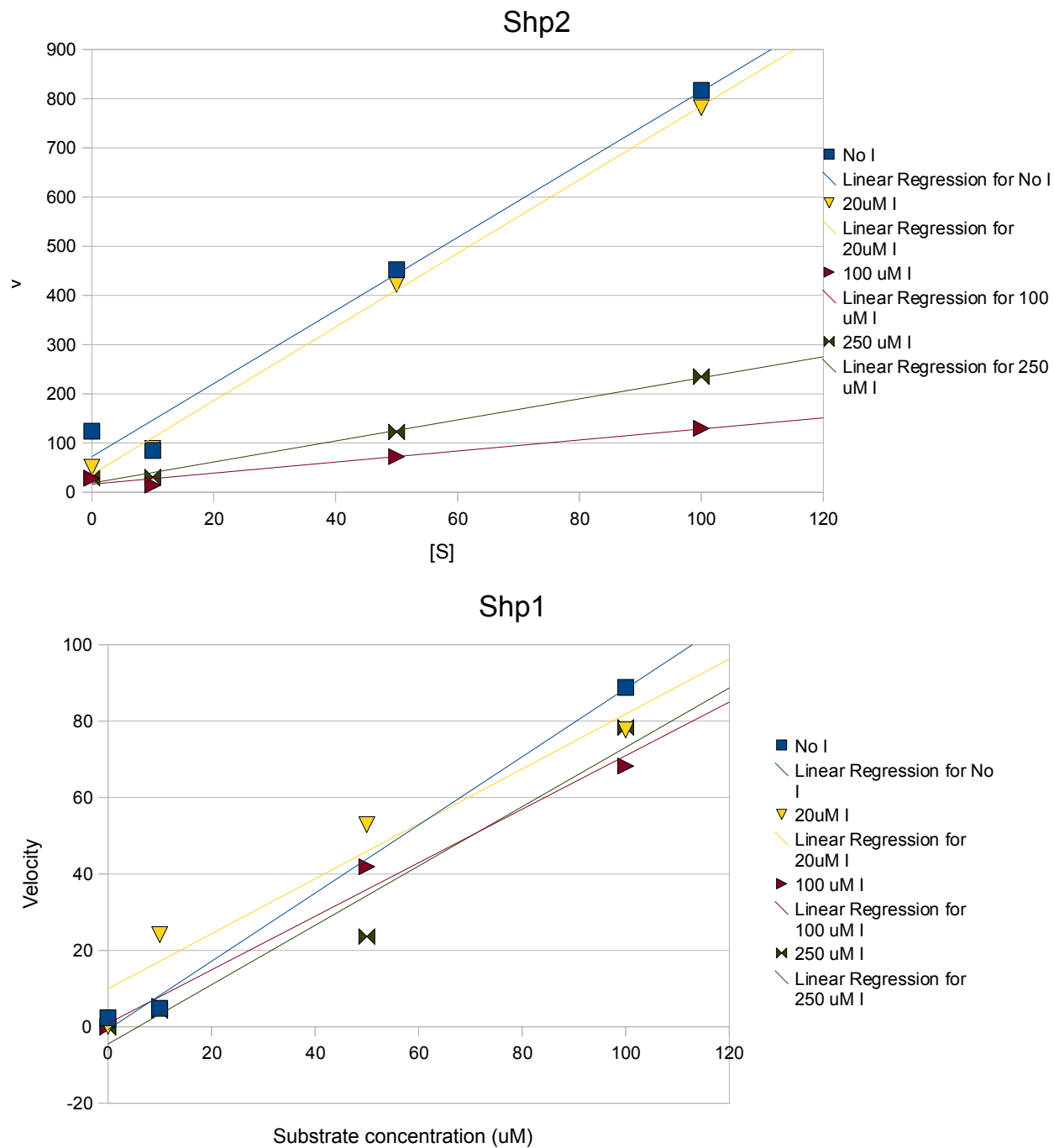
Shp2

Shp1

Figure 1. A kinetics assay using the phosphatase substrate difluoromethylumbelliferryl phosphate was followed at varying substrate concentrations in the presence of Shp2 or Shp1. Varied concentrations of inhibitor were also incubated in the reaction, and the production of fluorescent product was detected over 20 minutes. Reaction velocity vs. substrate concentration shows that the peptide shows a clear preference for Shp2 inhibition instead of Shp1 inhibition.

Peptide Sequence	Predicted energy change due to binding (kcal/mol)
DADEpYL	-124.2±11.8
DADEYL	-57.2±8.5
DADApYL	-108.3±10.3
DAAEpYL	-148.1±9.8
DAAApYL	-121.6±8

Table 1. Average predicted energy stabilization due to binding of the given peptides to the Shp2 active site. Data is the average of 10 separate 500 picosecond molecular dynamics simulations and was calculated using the MM_PBSA module of the AMBER9 software package.
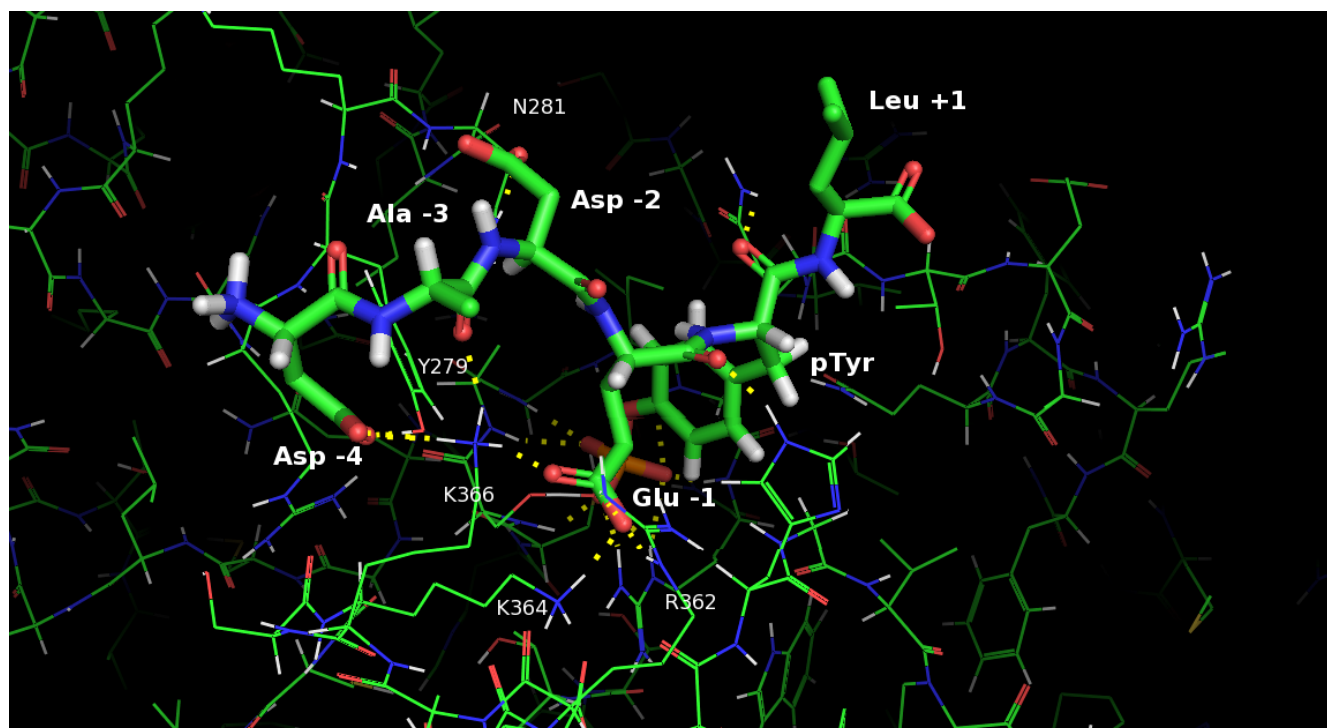
Figure 2. Conformation of peptide DADEpYL after 500 picoseconds of molecular dynamics. Shp2 residues are labeled in non-bold font, while ligands are labeled in bold and are numbered with respect to placement in the primary sequence in relation to the phosphotyrosine. Polar contacts are represented by yellow dashes. Prominent ionic interactions are found to form between the -1 position glutamate and the arginine 362 as well as lysine 364. The -4 aspartate forms strong contacts with lysine 366 and an ion-dipole interaction with the hydroxide of tyrosine 279. The -2 aspartate forms an ion-dipole interaction with asparagine 281, but it also interacts strongly with the water (not shown) due to its solvent accessibility.
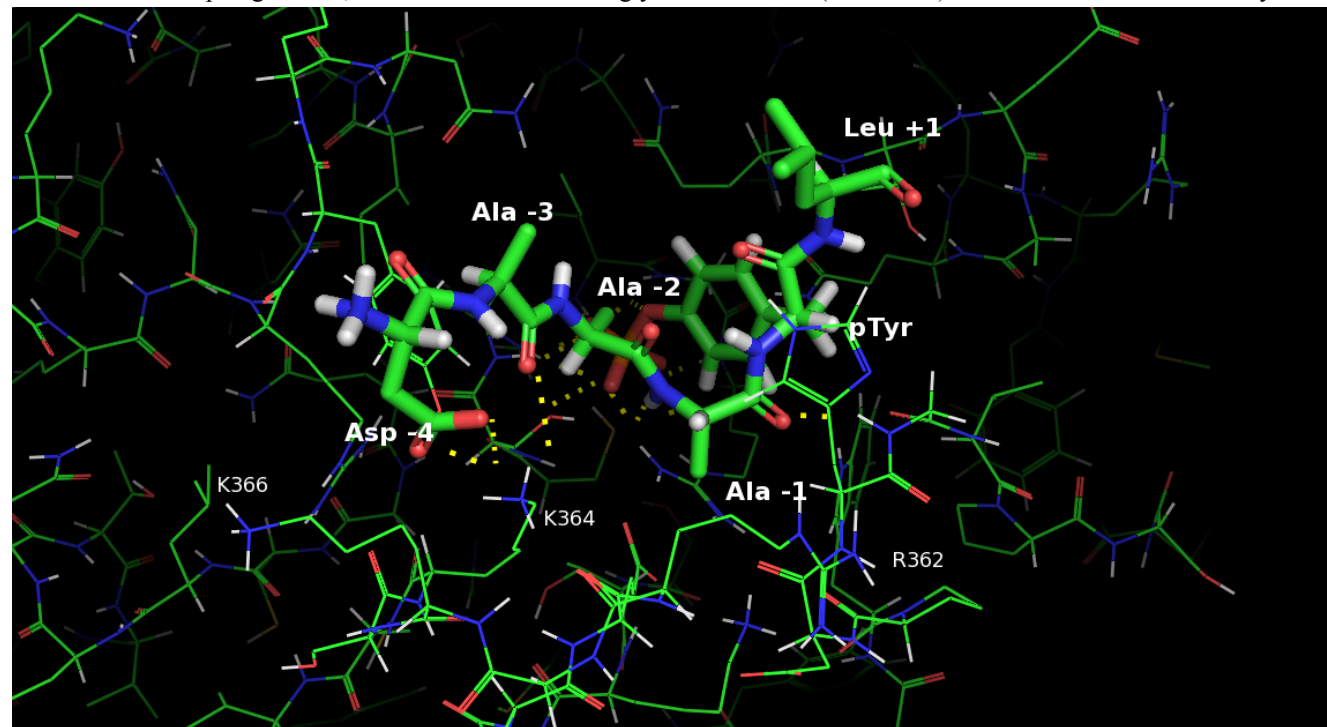

Figure 3. Conformation of peptide DAAApYL after 500 picoseconds of molecular dynamics. The -4 position aspartate forms ionic interactions with lysine 364. Note the displacement of lysine 366 and arginine 362 to positions where they cannot interact with the peptide, whereas in the DADEpYL wildtype peptide interactions with these residues are prominent.
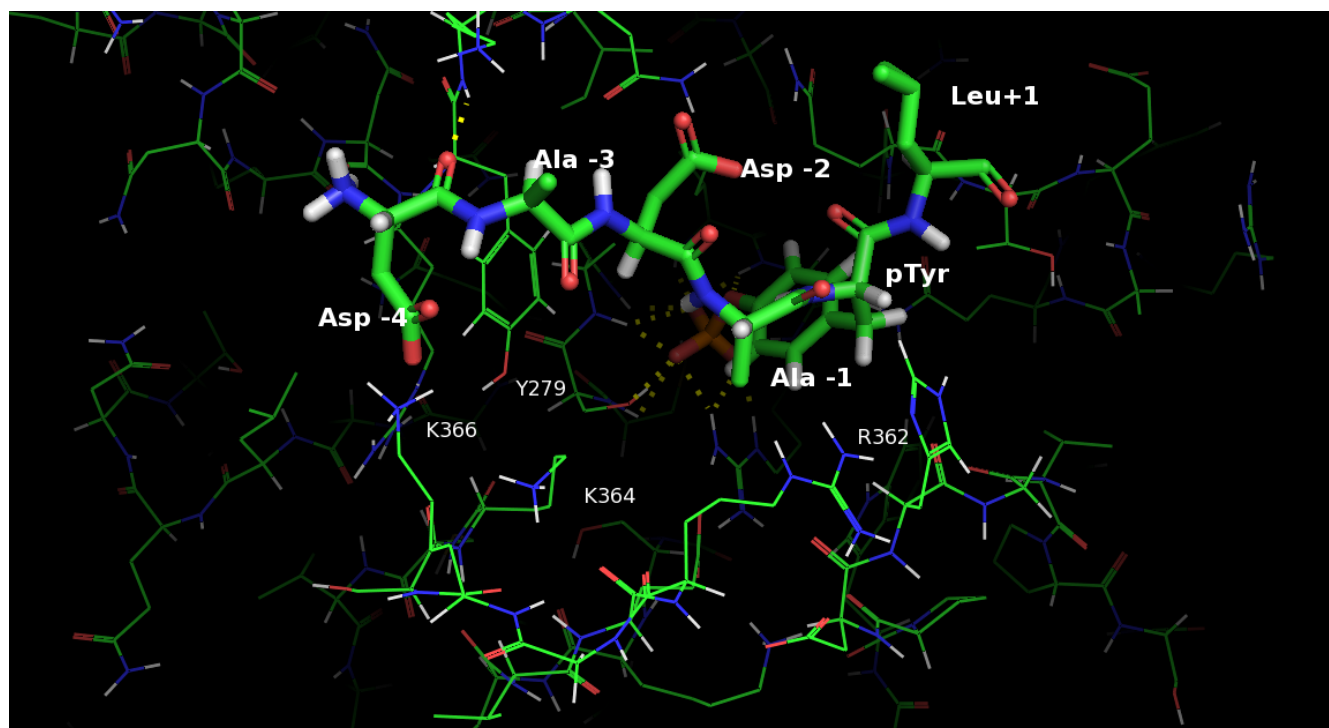
9

Figure 4. Conformation of peptide DADApYL after 500 picoseconds of molecular dynamics. No major polar contacts are found in this structure, which may help to explain the predicted binding defect. Note that the -2 position aspartate interacts significantly with water due to its solvent accessibility (not shown).
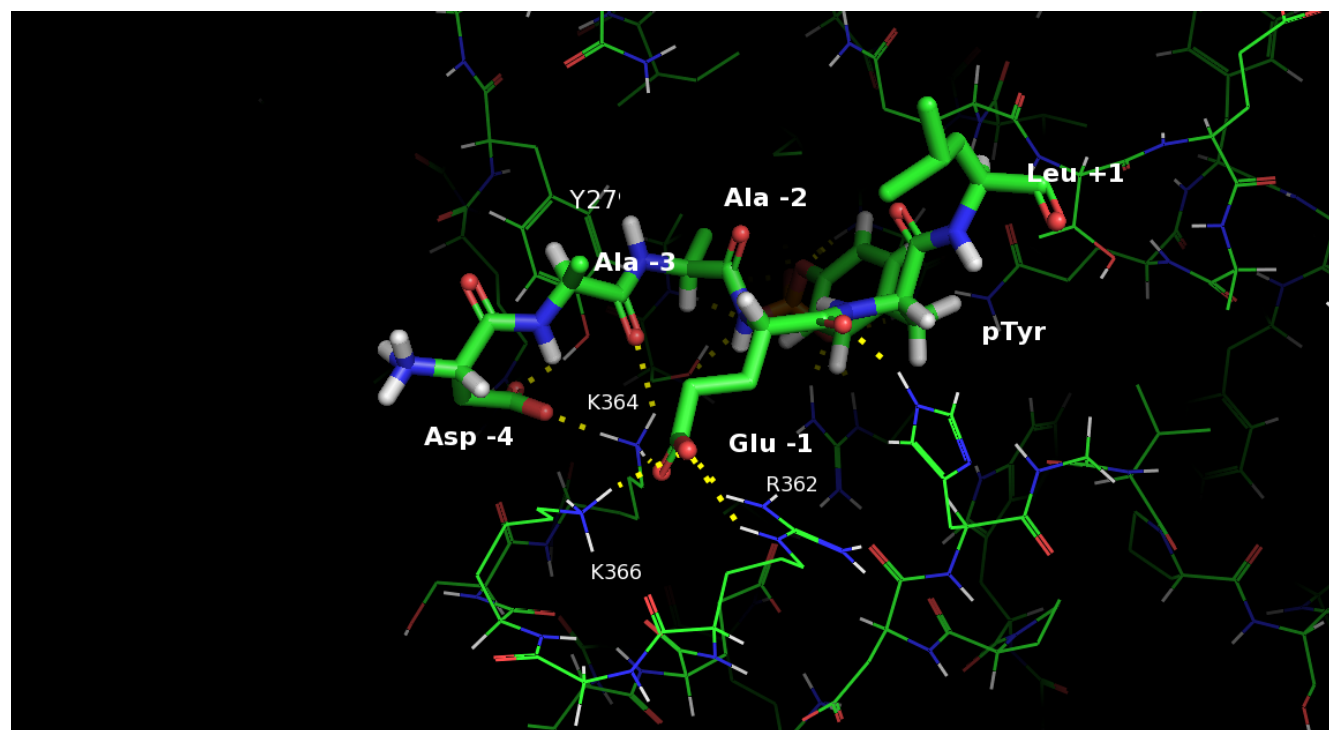

Figure 5. Conformation of peptide DAAEpYL after 500 picoseconds of molecular dynamics. This peptide was found to bind quite similarly to the wildtype peptide, with ionic interactions to lysines 364 and 366 and arginine 362. Interactions with tyrosine 279 are also present. The mutation from aspartate to alanine at the -2 position caused the sidechain to become more buried into the binding site instead of remaining solvent-accessible, placing the acidic residues in a more favorable binding location, which may explain the enhanced predicted binding of this peptide compared to the wildtype peptide.
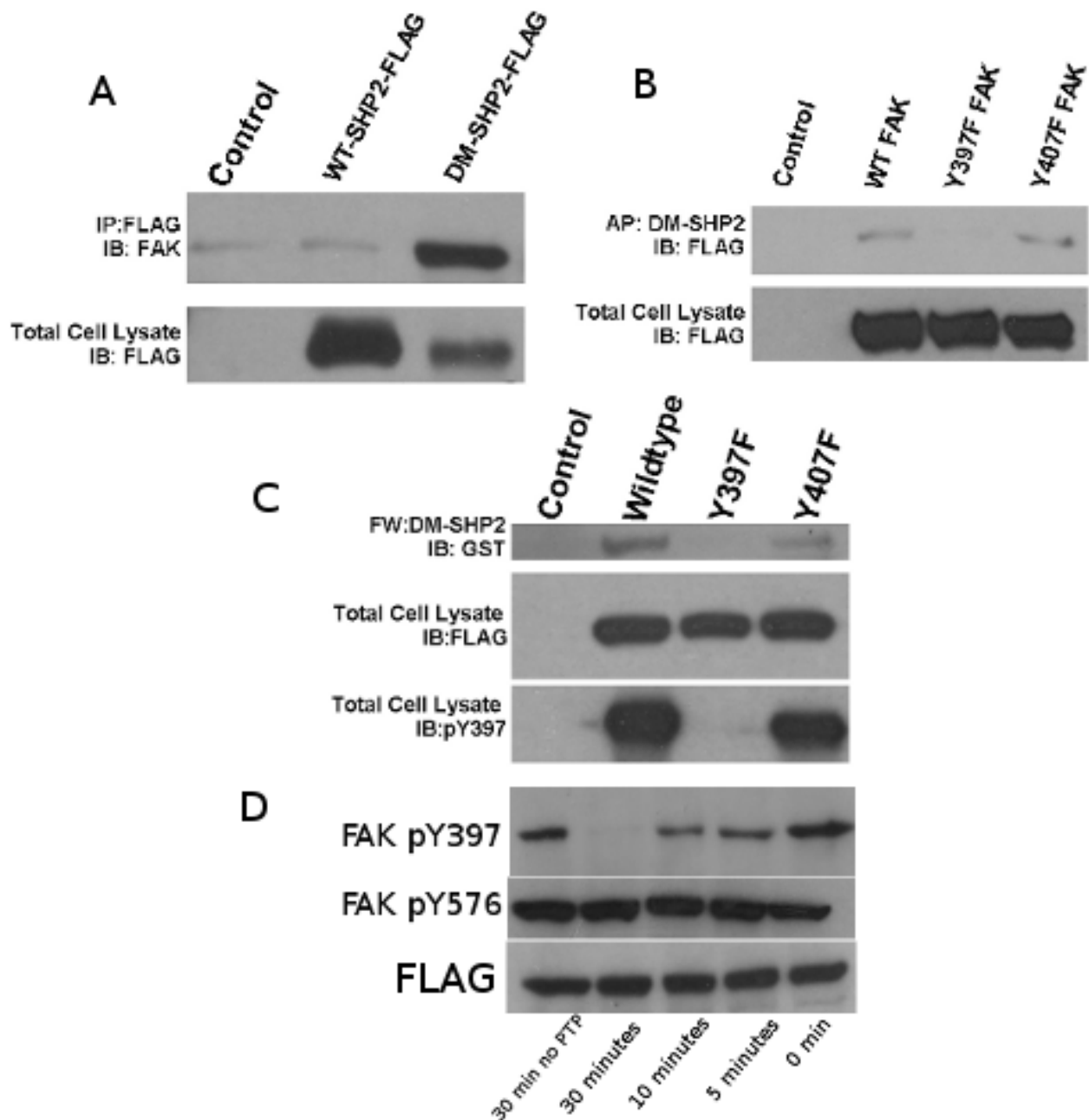
Figure 6. Focal adhesion kinase (FAK) is a substrate for Shp2.
A) Wildtype murine embryonic fibroblasts were transfected with FLAG-tagged wildtype or non-catalytic substrate-trapping (DM) Shp2. The cells were seeded on fibronectin before lysis and immunoprecipitation using anti-FLAG antibody. Immunoblotting for focal adhesion kinase reveals association of the two proteins.
B) Two phosphotyrosine sites that share close primary sequence homology (Y397 is ETDDYAEI, and Y407 is EEDTYTMP) were mutated to phenylalanine in an attempt to establish which phosphotyrosine regulates binding of Shp2 to FAK. Mutation of Y397 abrogated association of FLAG-tagged FAK with DM-Shp2.
C) In order to determine whether binding of Shp2 to FAK is direct or through binding of a different molecule in complex, Far Western analysis was performed whereby the cell lysate was immobilized on nitrocellulose before probing with recombinant GST-tagged DM-Shp2. Anti-GST was then used to detect the binding of Shp2 to bands on the membrane.
D) In vitro phosphatase assay was performed on wildtype FAK. Cell lysates from fibroblasts seeded on fibronectin were subjected to 1 ug recombinant Shp2 for the indicated time points and were then probed for anti-pY397 or anti-pY576. The lane labeled "30 min no PTP" was used as a negative control for endogenous phosphatase activity of the cell lysates.

11

Key Research Findings

- The DADEpYL peptide, as hypothesized, appears to show selectivity for Shp2 against Shp1
- Predicted binding from the initial computational docking of the DADEpYL peptide is in agreement with refined molecular dynamics simulations.
- The -2 position aspartate residue of the substrate-based peptide is predicted by simulations to impede binding of the peptide to Shp2, an effect which is abolished upon *in situ* mutagenesis to alanine.
- Focal adhesion kinase is a potential substrate of Shp2, predicted by the findings from the docking and dynamics simulations. The ability to predict new substrates allows us to dissect with greater detail the role of Shp2 in breast cancer as well as its potential as a therapeutic target.

Reportable Outcomes

Presentations supported by this award:

- Poster at the 2011 WVU Van Liere Research Convocation
  - Exploring the molecular mechanism of SHP2 regulation of cell motility and invasion
- Oral presentation at the 2011 WVU Van Liere Research Convocation
  - Exploring the molecular mechanism of SHP2 regulation of cell motility and invasion
- 2 posters presented at the 2011 American Association for Cancer Research San Antonio Breast Cancer Symposium
  - Inhibition of Shp2 Abolishes Mammary Tumorigenesis in Mice
  - Molecular mechanism for Src homology phosphotyrosyl phosphatase 2 regulation of cell motility and migration

Conclusion

The research characterized in this report demonstrates some previously unknown data regarding the nature of Shp2 binding its prototype substrate. Acidic residues N-terminal to the phosphotyrosine of a substrate appear to play a significant role in this binding, according to computational simulations. Most surprisingly, however, it appeared that position of acidic residues had a significant impact on the predicted interactions. In moving forward in predicting other Shp2 substrates, we can take into account this new information. The simulation data also adds some confirmation that interaction of acidic sidechains with lysines and arginines adjacent to the active site provide the majority of the binding stability. Since the binding region of Shp1 has a different distribution of these positively-charged amino acids, this work implies that binding to this region on Shp2 is the reason the enzyme is able to recognize substrates and compounds selectively. Accordingly, future compounds targeting Shp2 should take advantage of this fact, if it proves to be correct by our biochemical analysis moving forward.

The fact that our model facilitates the successful prediction of other Shp2 substrates suggests that the experiments are moving in the proper direction and allows us to better characterize Shp2 in cellular processes that are related to cancer. By understanding the biological functions and mechanisms of Shp2 action, we can better characterize it as an important therapeutic target in breast cancer.

References

Chen, L., Sung, S. S., Yip, M., Lawrence, H. R., Ren, Y., Guida, W. C., Sebti, S. M., et al. (2006). Discovery of a novel shp2 protein tyrosine phosphatase inhibitor. *Molecular pharmacology*, *70*(2), 562. ASPET. doi:10.1124/mol.106.025536.activation

Hellmuth, K., Grosskopf, S., Lum, C. T., Würtele, M., Röder, N., von Kries, J. P., Rosario, M., et al. (2008). Specific inhibitors of the protein tyrosine phosphatase Shp2 identified by high-throughput docking. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(20), 7275-80. doi:10.1073/pnas.0710468105

Inagaki, K., Noguchi, T., Matozaki, T., Horikawa, T., Fukunaga, K., Tsuda, M., Ichihashi, M., et al. (2000). Roles for the protein tyrosine phosphatase SHP-2 in cytoskeletal organization, cell adhesion and cell migration revealed by overexpression of a dominant negative mutant. *Oncogene*, *19*(1), 75-84. doi:10.1038/sj.onc.1203204

Manes, S., Mira, E., Gomez-Mouton, C., Zhao, Z. J., Lacalle, R. A., & Martinez-A, C. (1999). Concerted activity of tyrosine phosphatase SHP-2 and focal adhesion kinase in regulation of cell motility. *Molecular and cellular biology*, *19*(4), 3125. Am Soc Microbiol. Retrieved from http://mcb.asm.org/cgi/content/abstract/19/4/3125

Mohi, M. G., & Neel, B. G. (2007). The role of Shp2 (PTPN11) in cancer. *Current opinion in genetics & development*, *17*(1), 23-30. doi:10.1016/j.gde.2006.12.011

Reiske, H. R., Kao, S. C., Cary, L. a, Guan, J. L., Lai, J. F., & Chen, H. C. (1999). Requirement of phosphatidylinositol 3-kinase in focal adhesion kinase-promoted cell migration. *The Journal of biological chemistry*, *274*(18), 12361-6. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10212207

Ren, L., Chen, X., Luechapanichkul, R., Selner, N. G., Meyer, T. M., Wavreille, A.-S., Chan, R., et al. (2011). Substrate specificity of protein tyrosine phosphatases 1B, RPTPα, SHP-1, and SHP-2. *Biochemistry*, *50*(12), 2339-56. doi:10.1021/bi1014453

Zhang, X., He, Y., Liu, S., Yu, Z., Jiang, Z. X., Yang, Z., Dong, Y., et al. (2010). Salicylic Acid Based Small Molecule Inhibitor for the Oncogenic Src Homology-2 Domain Containing Protein Tyrosine Phosphatase-2 (SHP2). *Journal of medicinal chemistry*, *53*(6), 2482–2493. ACS Publications. doi:10.1021/jm901645u

Zhou, X, Coad, J., Ducatman, B., & Agazie, Y. M. (2008). SHP2 is up-regulated in breast cancer cells and in infiltrating ductal carcinoma of the breast, implying its involvement in breast oncogenesis. *Histopathology*, *53*(4), 389-402. doi:10.1111/j.1365-2559.2008.03103.x